Exhibit A

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Tubal Ligation Induces Quiescence in the Epithelia of the Fallopian Tube Fimbria

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Abstract

Tubal ligation keeps the fimbriated end of the fallopian tube intact while interrupting the conduit for sperm and egg between the uterus and ovary. Tubal ligation is associated with an approximately 20% decreased risk of high-grade serous ovarian cancers, which mounting evidence suggests arise from the distal fallopian tube epithelium. We postulated that biological changes at the epithelial cellular level of the distal fallopian tube may account for the surgical procedure's observed risk reduction. We compared the histology, presence of epithelial progenitors (basally located CD44-positive cells), and degree of epithelial proliferation (Ki67-positive cells) of distal fallopian tube from 10 patients with previous tubal ligation and 10 age-matched patients with uncut fallopian tubes. A significantly reduced population of proliferating epithelial progenitors (basally located CD44/Ki67 dual-positive cells) was detected in the tubal ligated specimens ($P = .0002$). To functionally assess the effect of tubal ligation, a murine model was utilized to compare the growth capacity of distal fallopian tube epithelial cells isolated from either ligated or sham-operated tubal epithelia. Murine fallopian tube epithelial cells isolated after tubal ligation showed a significantly reduced capacity to grow organoids in culture compared to sham-operated controls ($P = .002$). The findings of this study show that tubal ligation is associated with a reduced presence and decreased proliferation of progenitor cells in the distal fallopian tube epithelium. These compositional and functional changes suggest that tubal ligation induces quiescence of distal fallopian tube epithelial cells.

Keywords

fallopian epithelial progenitors, tubal ligation, fallopian tube epithelial proliferation

Introduction

Tubal ligation has sustained popularity as one of the most common methods of contraception for women in the United States since its peak usage in the 1970s.[1,2] Initially a surgical procedure that only involved laparotomy incisions, tubal ligation has evolved to be less invasive with the utilization of laparoscopic methods.[2] A laparoscopic tubal ligation may include cauterization or the application of clips or rings to the fallopian tube in order to block blood supply and cause scarring that blocks the passage of sperm or egg.[3] The fallopian tube may also be partially removed, such as with the Pomeroy technique, or fully removed via a salpingectomy.[3,4] Despite differences in approach, all these tubal ligation methods lead to gross morphologic changes in the fallopian tube to render the organ no longer functional. However, potential microscopic changes that may occur in the fallopian tube epithelium following a tubal ligation have not been widely investigated.

The fallopian tube is not just a passive conduit between the ovary and uterus to facilitate the passage of sperm and egg. The fallopian tube is an active organ that may undergo structural changes in response to the menstrual cycle as well as to menopause.[5] The epithelium has the capacity to proliferate and change its cellular composition in preparation for ovulation.[6] As a further demonstration of the organ's dynamic nature, surgical reversal of tubal ligation via reanastomosis may be effective in restoring a woman's ability to become pregnant by rehabilitating the function of the fallopian tube.[7-9] Depending on a multitude of factors including the remaining length of the fallopian tube and type of tubal ligation performed, surgical reanastomosis has pregnancy success rates ranging from 25% to 83%.[7,8] The success of surgical reanastomosis to recover function following tubal ligation associated scarring and

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blockage of blood flow indicates that the fallopian tube has regenerative capacity at the cellular level.[10] As early descendants of stem cells that can differentiate into different cell types, progenitor cells enable a tissue to repair itself and may thus likely account for the fallopian tube's regenerative function.[11] Previous work in our laboratory has shown that progenitor cells reside along the fallopian tube from the proximal to distal end and are concentrated in the fimbriated epithelium.[11] The presence of progenitor cells throughout the fallopian tube may account for the organ's regenerative capacity following a surgical reanastomosis performed at any site.

Recent evidence strongly suggests that many serous ovarian carcinomas arise from the distal fallopian tube rather than from the ovarian surface epithelium.[12-16] High-grade serous ovarian tumors are the most prevalent and deadly histotype of epithelial ovarian cancers, which are among the most aggressive and deadly gynecologic malignancies.[17-19] In a pooled analysis of case–control studies, women with a history of tubal ligation had a reduced risk of invasive serous cancer by 19%.[20] This procedure is also inversely associated with the development of endometrioid, clear cell, and mucinous carcinomas with a risk reduction of 32% to 52%.[20] Although several mechanisms have been proposed to explain tubal ligation's observed risk reduction of serous tumors, the existing literature does not specifically examine the possibility that changes at the tissue level of the fallopian tube epithelium may contribute to this risk reduction.

Mechanisms that have been proposed to explain risk reduction of epithelial ovarian cancers by tubal ligation include the prevention of ascending carcinogens (eg, talc) through the Müllerian system toward the ovaries, possible changes in ovarian function, and potential development of protective antibodies. Two population-based analyses have not demonstrated any clear association between exposure to talc use and ovarian cancer development.[21,22] These studies suggest that interruption of the ascent of carcinogens by tubal ligation is unlikely to be a significant contributing factor to the procedure's risk reduction. Moreover, although existing studies have reported lower hormone levels in women postligation compared to women without tubal ligation, they did not examine preligation hormone levels (reviewed in[23]). This makes it difficult to draw any conclusion regarding the effect of tubal ligation on ovarian function as a possible explanation for the risk reduction of ovarian cancer. Another proposed theory is that increased anti-Mucin-1 (MUC1) antibodies seen in women with tubal ligation may have a protective effect against ovarian cancer in younger women.[24,25] Although this is an interesting population-based observation, the functional role of anti-MUC1 antibodies in the prevention of ovarian cancer remains to be investigated.

The greatest risk reduction following tubal ligation among the various epithelial ovarian cancers is observed for endometrioid and clear cell ovarian tumors.[20] A suggested theory for the risk reduction in these 2 histologic subtypes is that tubal ligation prevents the migration of ascending cells originating in the endometrium from reaching and implanting onto the ovary by interrupting the conduit.[14,23] However, this theory does not shed light on the mechanism underlying the risk reduction of high-grade serous cancers that are thought to arise from the distal fallopian tube, which remains intact following tubal ligation.

We hypothesized that changes in the cellular composition and activity of the distal fallopian tube epithelium following tubal ligation may explain why there is a risk reduction of serous cancers associated with this surgical procedure. Since the fimbriated end of the fallopian tube is left intact following tubal ligation and is the proposed site for serous cancer initiation, our study focused on analyzing the presence and activity of distal fallopian tube epithelial progenitor cells. Through analyses of human patient samples and in vitro growth assays using a murine model, here we investigate whether tubal ligation leads to quiescence of distal fallopian tube epithelium.

Materials and Methods

Patient Cohorts

This retrospective study was approved by the University of California Los Angeles (UCLA) Office for the Protection of Research Subjects (IRB 12-001213). Of approximately 600 reviewed medical records of patients that had undergone salpingectomy at UCLA in the past 10 years, 580 were excluded from our study due to either (1) history of any malignancy including gynecologic cancers, (2) any hormone replacement therapy including oral contraceptives since exogenous hormones may affect cellular composition of the fallopian tube, or (3) unavailability of histologic sections containing distal fallopian tube. This study consists of 2 cohorts with equal age distributions: 10 patients with a history of tubal ligation and 10 age-matched controls (Table 1). Both pre- and postmenopausal patients were selected for this study. An expert gynecologic pathologist (PS) reviewed all samples and determined them to be normal and without any evidence of pathology.

Immunohistochemistry

Formalin-fixed and paraffin-embedded histologic sections were stained with hematoxylin and eosin to observe tissue morphology. Paraffin sections were prepared for immunohistochemistry through a process of heat-induced epitope retrieval as previously described.[11] Both the control and tubal ligation cohorts were immunostained for CD44 and Ki67 with antibodies and substrates listed in Supplementary Table 1. Staining quality and specificity for CD44 and Ki67 were evaluated against serous ovarian tumor as a positive control (high CD44 and Ki67 expression) and endometrial myometrium as a negative control (minimal CD44 and Ki67 expression). Clonal epithelial organoids regenerated from mouse fallopian tube epithelia were prepared in a similar fashion and immunostained for 2 antigens that mark the differentiated cells of the fallopian tube: Pax8, a transcription factor marking secretory cells,[26,27] and β-tubulin, a structural protein that marks ciliated cells.[28] Endometrial stroma was used as a negative control for Pax8

Table 1. Patient Cohorts.

Patient	Age	Indication for Surgery
A. Tubal ligation cohort		
1	31	Pelvic pain, menorrhagia, ruptured luteal cyst
2	54	Benign ovarian cyst
3	35	Benign cystadenoma
4	50	Benign fibrothecoma
5	62	Benign cystadenoma
6	53	Benign cystadenoma
7	37	Benign sclerosing stromal tumor
8	38	Benign hemorrhagic corpus luteum cyst, BRCA+
9	50	Hemorrhagic luteinized cyst
10	62	Risk-reducing bilateral salpingo-oophorectomy
B. Control cohort		
1	34	Teratoma
2	54	Adenomyosis
3	35	Benign cystic follicles
4	50	Benign cystadenoma
5	62	Benign mucinous cystadenoma
6	53	Prolapse, stress urinary incontinence
7	34	Simple serous ovarian cyst
8	40	Risk-reducing salpingo-oophorectomy, BRCA+
9	50	Adenomyosis
10	62	Benign fibrothecoma

and β-tubulin staining. Human fallopian tube was used as a positive control for the Pax8 and β-tubulin stains.

Stained slides were submitted to the Translational Pathology Core Laboratory, a research facility in the UCLA Department of Pathology and Laboratory Medicine, for digital imaging. Using Aperio ScanScope linear-array scanning technology, high-quality scans of each slide were obtained at 20-fold magnification.[29] Scanned samples were then analyzed using Definiens Tissue Studio, in which unique solutions were constructed for each stain in order to identify positively stained cells and to obtain a total cell count.[30] For the single and dual stains, the total numbers of CD44-positive, Ki67-positive, and CD44/Ki67 dual-positive basal epithelial cells were manually verified independently by 2 researchers.

Images of the stains seen in the figures were produced on an Olympus BX51 upright microscope (Olympus, Melville, New York, http://www.olympusamerica.com) equipped with Optronics macrofire charge-coupled device camera and Optronics PictureFrame software.

Animals

Wild-type (C57BL/6), green transgenic (C57BL/6-Tg[ACTbEGFP]1Osb), and red transgenic (C57BL/6-Tg[ACTBDsRed.MST]1Nagy/J) mice were obtained from Jackson Laboratory. Color-marked mice were only used for clonal assays related to the formation of fallopian tube organoids. For all other assays, wild-type mice were utilized. Mice were maintained in accordance with Division of Laboratory Animal Medicine guidelines at UCLA. All animal experiments were approved by the Animal Research Committee at UCLA.

In Vitro 3-Dimensional Organoid Assay of Fallopian Tube Epithelia

Microscopic tubal ligations mimicking a modified Pomeroy method and sham operations were performed on 6- to 8-week-old reproductive wild-type mice, followed by a 4-week recovery period. Distal fallopian tubes of 5 mm were harvested from both the sham and tubal ligation cohorts. This measurement ensured that an equal amount of fallopian tube was utilized in both experimental groups. These tubes were cut into fragments and suspended in Dulbecco modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS). They were then washed in phosphate-buffered saline and 5 mmol/L EDTA, incubated at 4 C for 45 minutes in 1% trypsin, then stopped with DMEM plus 10% FBS. Distal fallopian tube epithelial cell sheets were mechanically isolated from fragments using two 25-gauge needles and were then incubated at 37 C in 0.8 mg/mL collagenase in DMEM and 5 μg/mL insulin for 1 hour 30 minutes. Cells were harvested, washed, and resuspended in 0.5% trypsin-EDTA for 5 minutes before being dissociated into single cells using a syringe. Dissociated cells were incubated on ice for 15 minutes with antibodies in preparation for sorting (Supplementary Table 2). Fluorescence activated cell sorting was utilized to isolate all epithelial cells that expressed the epithelial cell marker epithelial cell adhesion molecule (EpCAM). Cells expressing lineage markers TER119 (red blood cells), CD45 (hematopoietic cells), and CD31 (endothelial cells) were excluded. Five thousand cells were then plated in 1:1 PREGM Matrigel and cultured for 11 days in an organoid assay as previously described.[11]

Statistical Methods

Median percentages of positive cells per antigen (CD44 and Ki67) were calculated from the total number of distal fallopian tube epithelial cells. For the CD44/Ki67 dual stains, median percentages of CD44/Ki67 dual-positive cells were calculated from the total number of CD44-positive basally located distal fallopian tube epithelial cells. Because the distribution of the antigens did not necessarily follow the normal distribution with constant variance, *P* values were computed using the nonparametric Wilcoxon rank sum test. Mean values of the number of organoids were compared between ligation and no ligation groups using a two-by-two repeated measure analysis of variance model. The criterion for statistical significance among all comparisons was set at an α of .05.

Results

Epithelia of Ligated Fallopian Tubes had a Lower Percentage of Basal Progenitors in the Fimbriated End Compared to Nonligated Samples

Previous work by this laboratory has shown that CD44 is expressed by a population of basally located epithelial cells with progenitor activity present throughout the fallopian tube

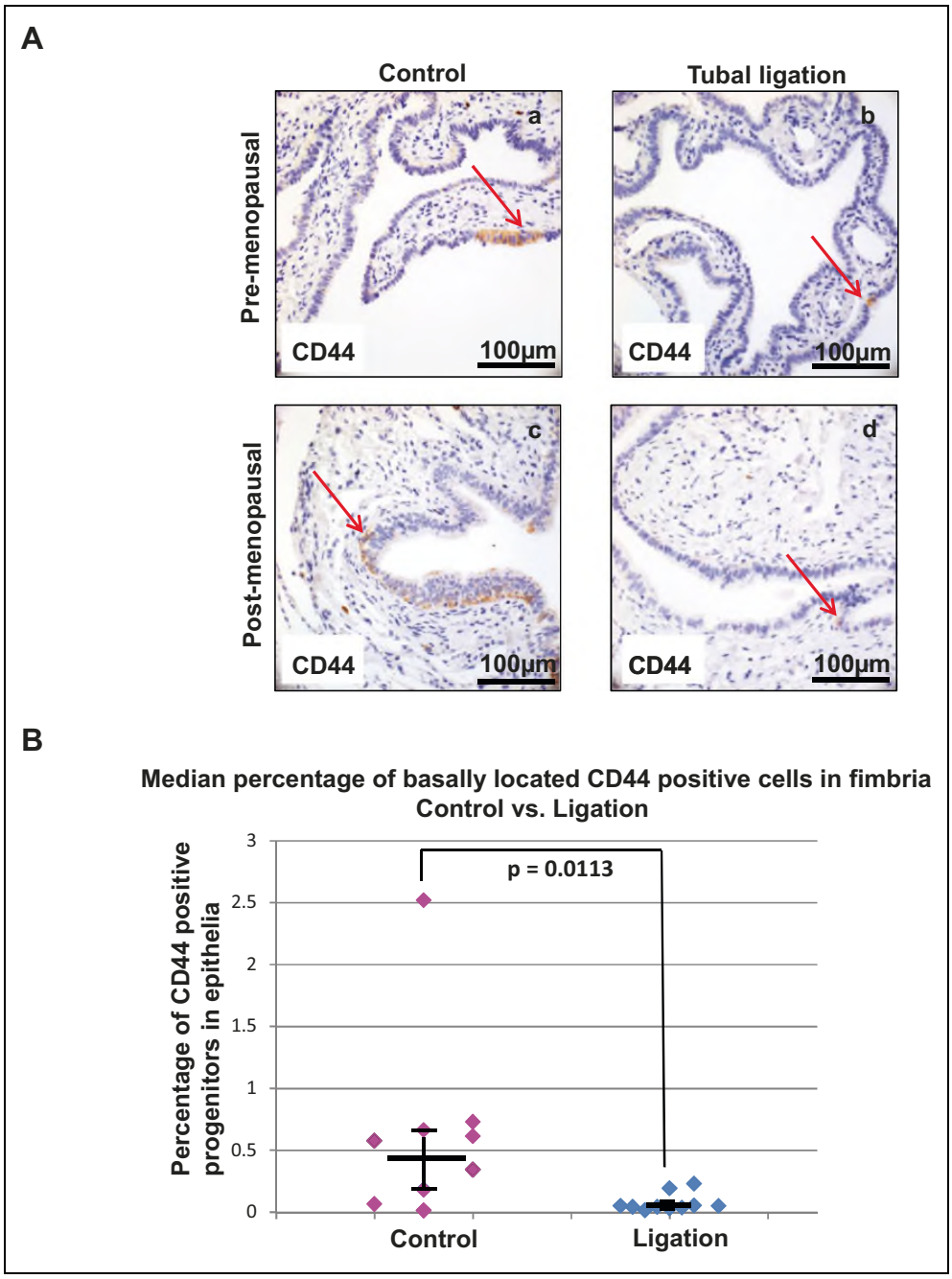

Figure 1. A lower number of progenitors was detected in the distal fallopian tube epithelium of patients who underwent tubal ligation. (A) Immunohistochemistry demonstrated the representative distribution of CD44 expression in the fimbria of intact fallopian tubes (a and c) versus ligated patient samples (b and d). A lower number of basally located CD44-positive cells was seen in both pre- (a vs b) and postmenopausal (c vs d) tubal ligation patient samples. Arrows point to individual CD44-positive basal epithelial cells. (B) The median percentage of distal fallopian tube epithelial progenitors (basally located CD44-positive epithelial cells) was reduced with tubal ligation. Dot plot summarizes and compares data points of all clinical samples, confirming a statistically significant difference at $P = .0113$. Horizontal bars represent the median for each cohort and the vertical bars denote interquartile range.

and concentrated in the distal fimbria.[11] Here we examine whether tubal ligation is associated with a change in the number of these progenitor cells specifically in the fimbria. Sections of distal fallopian tube epithelia from patients that had undergone tubal ligation and aged-matched controls were stained for CD44 (Figure 1A). Although no obvious histologic differences were observed between the ligated and nonligated human fallopian tube samples, the fimbriated fallopian tube epithelium of patients with previous tubal ligation had an approximately 9-fold decrease in the median percentage of progenitor epithelial cells compared to that of patients without tubal ligation. The epithelial lining of the tubal ligation cohort contained 0.05 median percent basal CD44-positive progenitors compared to the 0.46 median percent seen in control

samples ($P = .0113$; Figure 1B). This suggests a significant reduction in progenitors in the distal fallopian tube epithelium with tubal ligation.

Tubal Ligation is Associated With Decreased Proliferation in the Progenitor Cells of the Fimbriated Fallopian Tube

Increased proliferation as measured by Ki67 expression has been associated with the progression from normal tissue to dysplasia to malignancy in the Müllerian duct epithelium.[31] It has also been shown that the expression of Ki67 can be a biomarker of aggressive behavior in tumors and may impact prognosis of disease.[32,33] Even in preneoplastic tissue, a high level of Ki67 expression may portend an increased risk of developing malignancy at a later time.[34] For example, a study of breast tissue found that a higher Ki67 index correlated with a significantly increased risk of developing invasive breast cancer in women with a diagnosis of atypical hyperplasia.[34] These observations imply that the Ki67 index may be used as a surrogate measure of a tissue's risk for becoming dysplastic.

Distal fallopian tube specimens from the tubal ligation and control cohorts were immunostained for Ki67 (Supplementary Figure 1A). Although the control group had a median Ki67 index of 0.44%, patients with tubal ligation had a median index of 0.14% ($P = .0140$; Supplementary Figure 1B). Decreased Ki67 expression indicated that the proliferation in the distal fallopian tube epithelium was significantly reduced in patients with tubal ligation compared to normal controls.

To investigate whether tubal ligation specifically affected the proliferation of the progenitor cells, histologic sections of distal fallopian tubes from patients with previous tubal ligation and their age-matched controls were dual stained for CD44 and Ki67 expression (Figure 2A). Although 16% of basally located CD44 cells stained positive for Ki67 in the control group, only 3% of basal CD44 cells were dual positive for Ki67 in the tubal ligation cohort (median values; $P = .0002$; Figure 2B). This indicated a 5-fold reduction in the proliferative progenitor cells of the distal fallopian tube epithelium following tubal ligation. Within both control and ligation cohorts in our study, the median percentages of basal CD44/Ki67 dual-positive epithelial cells was not significantly different between the pre- and postmenopausal patients ($P > .05$; Supplementary Figure 2). Collectively, we observed changes in both the composition and the proliferative index of epithelial progenitor cells in the distal fallopian tube when comparing age-matched cohorts of patients who had intact or ligated fallopian tubes.

Tubal Ligation Reduced In Vitro Growth Capacity of Murine Distal Fallopian Tube Epithelia

The decreased number and lower proliferation of progenitor cells detected in the human distal fallopian tube epithelia was correlational and may have been confounded by small sample size. Work by other investigators demonstrated that similar to the human tubal epithelium, mouse fallopian tubes also contain a progenitor stem-like population.[35,36] Therefore, here a murine model was utilized to functionally test the effects of tubal ligation on the growth capacity of distal fallopian tube epithelia to determine whether the observations seen in the human samples correspond to biological changes in the tubal epithelium. Two cohorts of 10 reproductive mice (aged 6-8 weeks) each underwent either tubal ligation using a surgical technique that mimics a modified Pomeroy method or sham operation. In the modified Pomeroy method, 2 independent knots were placed in the ampullary region of the fallopian tube to allow excision of an approximately 5 mm portion while leaving the proximal and distal regions of the fallopian tube intact. After 4 weeks of recovery time, 5 mm sections of the distal fallopian tube were harvested from both cohorts. Using methods previously established in our laboratory,[11] fallopian tube epithelium was isolated based on expression of EpCAM through a cell sorter. These epithelial cells were then plated in an in vitro 3-dimensional organoid growth assay that allowed for the quantification of individual epithelial cells with the capacity to give rise to fallopian tube epithelial organoids (Supplementary Figure 3A).[11] Fallopian tube organoids were clonal in origin (arose from a single epithelial cell) and histologically resembled normal fallopian tube epithelium based on expression of epithelial markers Pax8 (secretory cells) and β-tubulin (ciliated cells; Supplementary Figure 3B and C). Equal numbers of enumerated epithelia isolated from mice with tubal ligation or sham operation were plated in the in vitro organoid assay. After 11 days, growth capacity was assessed as percentage of epithelial cells that gave rise to organoids (Figure 3A).

In 2 independent repeats of this experiment, an approximately 2-fold reduction in the number of tubal epithelial cells with regenerative capacity was detected upon tubal ligation with 1.6% of epithelial cells giving rise to organoids in ligated mice versus 3% in the sham-operated cohort ($P = .002$; Figure 3B). This functional analysis mirrors the findings seen in the human specimens where a reduction in epithelial progenitors of fimbriated fallopian tube was seen in patients with tubal ligation compared to controls. Findings here using the mouse model demonstrated that tubal ligation decreased the overall growth capacity of fimbriated epithelium.

Discussion

Pathologic changes in the fallopian tube following tubal ligation have been documented.[10] Distortion and loss of musculature, thickening of epithelial folds, and dilation of the lumen of the remaining fallopian tube contribute to the organ's loss of function following the surgical procedure.[10] To our knowledge, however, it has not been greatly investigated how tubal ligation may lead to changes at the cellular level of the fallopian tube epithelium. We found that tubal ligation is associated with both a decreased number and reduced proliferative index of epithelial progenitors in the distal fallopian tube. Our observations of a decreased number and proliferation of progenitor cells in human distal fallopian tube specimens were corroborated by in vitro growth assays in a murine model that showed that distal

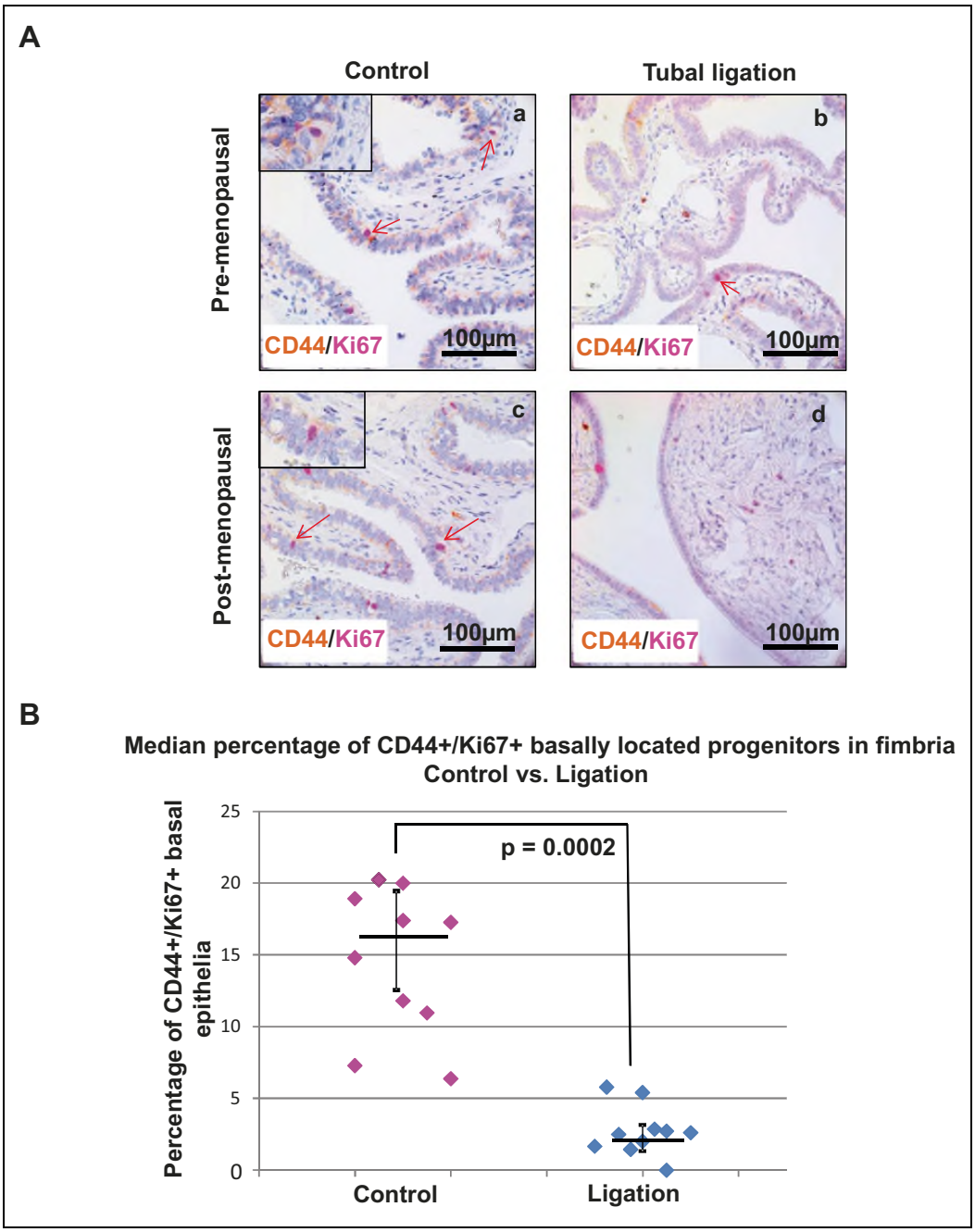

Figure 2. The percentage of proliferating epithelial progenitors was diminished in the distal fallopian tube of patient samples with tubal ligation. (A) Immunohistochemistry revealed a lower expression of proliferating progenitors (basally located CD44/Ki67 dual-positive epithelial cells) in the distal fallopian tube of ligated patient samples (b and d) compared to intact fallopian tubes (a and c). Light brown staining corresponds to CD44 expression and magenta staining indicates Ki67 nuclear expression. Tubal ligation samples from both pre- (b) or postmenopausal (d) patients showed a reduction in CD44/Ki67 dual expression compared to the control cohort. (B) The median percentage of proliferating progenitors was significantly lowered in the fimbria of tubal ligated patient specimens compared to age-matched controls at $P = .0002$. Each dot on the chart represents the percentage of basally located CD44-positive epithelial cells that also expressed Ki67. Horizontal bars represent the median for each cohort and the vertical bars denote interquartile range.

fallopian tube epithelial cells have a decreased growth capacity following tubal ligation. Similarly, work by others has demonstrated that following a surgical procedure, changes do occur in the composition and proliferation in the epithelia of the colon, a tubular organ containing an intraepithelial lining similar to the fallopian tube.[37,38] In these studies, a substantial decrease in number and mitotic activity of cells in the progenitor region of the colonic crypts was detected when comparing rats that had underwent a colostomy procedure to those with sham operations.[37,38]

Since our findings suggest that the epithelium is less active in proliferation following tubal ligation, we think the surgical

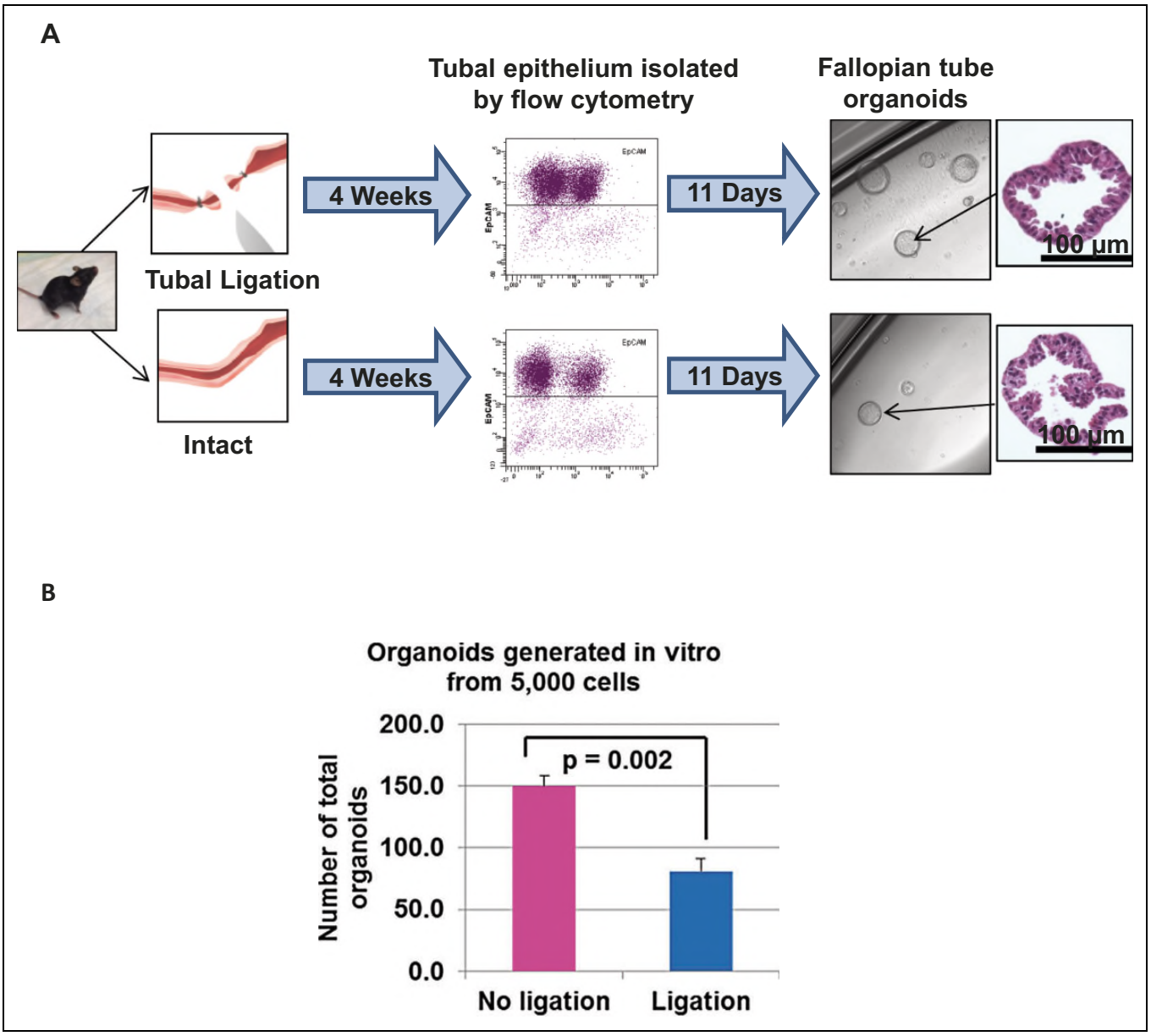

Figure 3. Epithelial cells residing in the fimbriated end of ligated fallopian tubes had a decreased capacity for forming in vitro organoids in a murine model. (A) Experimental schema for isolation and in vitro 3-dimensional growth of fallopian tube epithelia from both ligated and intact murine fallopian tubes. (B) Epithelia obtained from ligated murine fallopian tubes showed a significant decrease in number of cells capable of organoid formation compared to intact tubes. Cumulative results from 2 independent experiments are shown with mean ± standard deviation (SD).

procedure causes the distal fallopian tube epithelial progenitors to become quiescent. Given that high-grade serous tumors can initiate in the distal fallopian tube epithelium,[12-16] quiescence of the epithelial progenitors in the fimbriated end of the fallopian tube may be one mechanism accounting for the risk reduction of serous cancer following tubal ligation. Recent evidence investigating squamous carcinomas demonstrated that tumorigenesis only begins when hair follicle stem cells are released from quiescence.[39] Work from these researchers suggests that mechanisms that maintain the quiescent state prevent these cells from being susceptible to loss of tumor suppressor genes or gain of oncogenes.[39] One can imagine that less active cell cycling of fallopian tube epithelial progenitors may play a protective role by reducing the frequency of cumulative genetic changes that can initiate disease. Such genetic changes may include functional inactivation of tumor suppressor genes BRCA1, BRCA2, and p53 that are seen in many serous cancers.

We think tubal ligation protects against epithelial ovarian cancers in a potential 2-pronged mechanism: (1) it interrupts the conduit and halts the migration of ascending endometrial cells from implanting on the ovary and developing into endometrioid and clear cell cancers as others have described[23] and (2) work in this article suggests that tubal ligation leads to

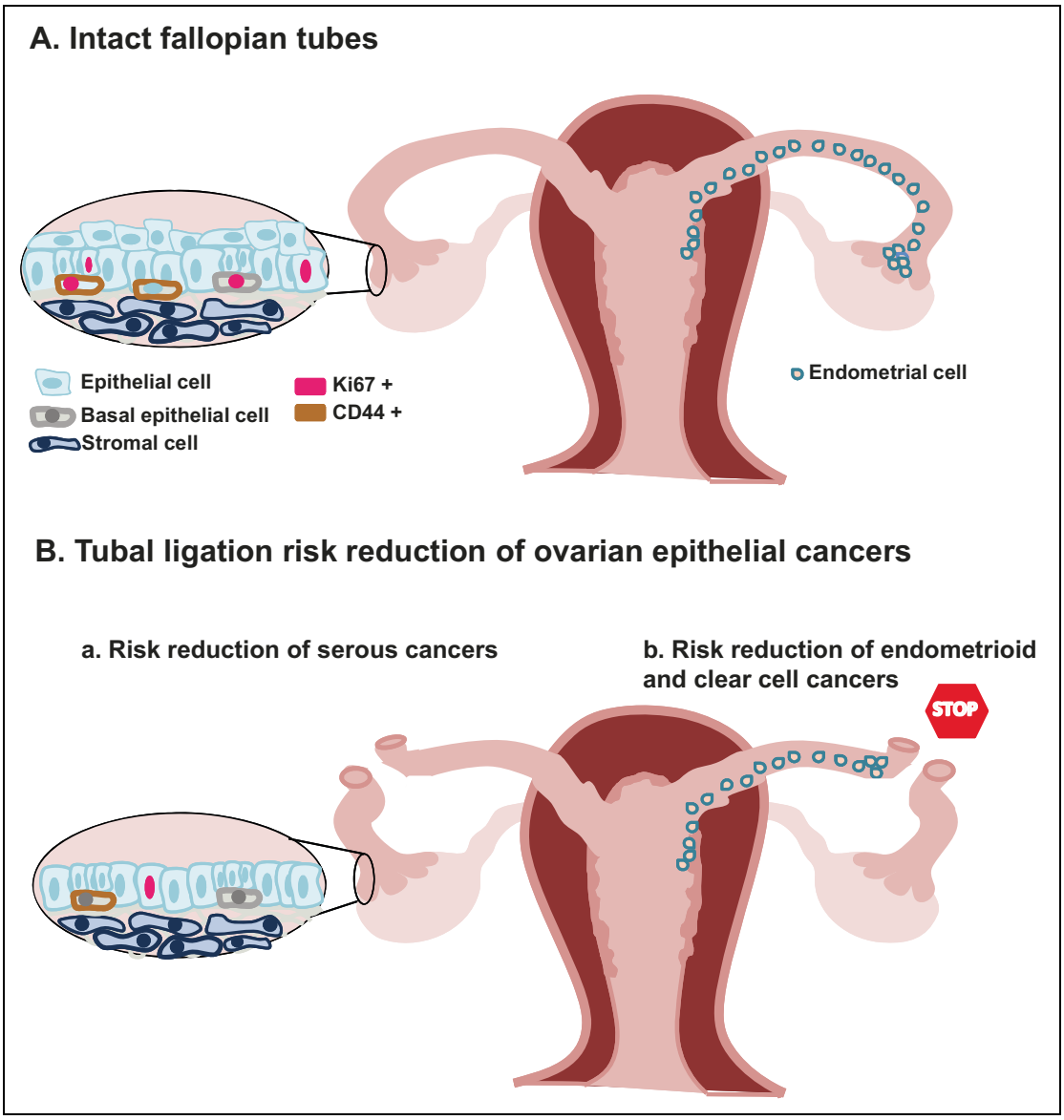

Figure 4. A model for risk reduction in epithelial ovarian tumors following tubal ligation. (A) We propose that the progenitors of the distal fallopian tube are normally cycling. This may allow for accumulation of genetic mutations that lead to serous cancer initiation, such as inactivation of tumor suppressors BRCA1, BRCA2, and p53. It has been proposed by other studies that when the fallopian tube is left intact, ascending endometrial cells are able to migrate and implant onto the ovary, which may lead to the development of endometrioid and clear cell cancers. (B) Our findings suggest that tubal ligation induces a state of quiescence in epithelia of distal fimbria by leading to a decreased population of progenitor cells with a lower proliferative index. A diminished number of proliferating progenitors may lower the frequency of cumulative genetic changes associated with serous cancer. As others have previously proposed, tubal ligation may prevent the migration of endometrial cells from initiating endometrioid and clear cell cancers on the ovary by interrupting the conduit between the uterus and ovary.

compositional and functional changes in the distal fallopian tube epithelium that renders it quiescent and possibly less likely to initiate serous carcinomas (Figure 4). Our findings reported here provide the basis for further validating these observations in a larger cohort of patients, which may require a multicentered analysis.

Although our study did not specifically explore mechanisms that can lead to quiescence in the distal fallopian tube epithelia, we think that 2 possible mechanisms based on review of literature may include changes in oxygenation of the fallopian tissue due to alterations in microvasculature[40-42] and/or decreased exposure to soluble cytokines and growth factors following tubal ligation.[43] A continuation of this study focused on mechanisms that regulate growth and regression of fallopian tube epithelial progenitor cells could shed insight on the factors that regulate their proliferation versus quiescence.

Authors' Note

Ekaterina Tiourin and Victor S. Velasco contributed equally to the work.

Declaration of Conflicting Interests

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Supplemental Material

The online data supplements are available at http://rs.sagepub.com/supplemental.

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Exhibit B

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Chronic Inflammation

Inflammation is a normal physiological response that causes injured tissue to heal. An inflammatory process starts when chemicals are released by the damaged tissue. In response, white blood cells make substances that cause cells to divide and grow to rebuild tissue to help repair the injury. Once the wound is healed, the inflammatory process ends.

In chronic inflammation, the inflammatory process may begin even if there is no injury, and it does not end when it should. Why the inflammation continues is not always known. Chronic inflammation may be caused by infections that don't go away, abnormal immune reactions to normal tissues, or conditions such as obesity. Over time, chronic inflammation can cause DNA damage and lead to cancer. For example, people with chronic inflammatory bowel diseases, such as ulcerative colitis and Crohn disease, have an increased risk of colon cancer.

Many studies have investigated whether anti-inflammatory medications, such as aspirin or non-steroidal anti-inflammatory drugs, reduce the risk of cancer. However, a clear answer is not yet available. For more information, see Aspirin to Reduce Cancer Risk.

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Exhibit C

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Inflammation is a key contributor to ovarian cancer cell seeding

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The incidence of ovarian cancer dramatically increases in early menopause but the factors contributing to cancer onset are unclear. Most ovarian cancers originate in the fallopian tube with subsequent implantation of malignant cells into the ovary. However, the events and conditions that lead to cancer cell implantation are unknown. To quantify which conditions are conducive to the seeding of cancer cells in an immunocompetent mouse model, we surgically implanted mouse ovarian cancer cells into the oviducts of syngeneic mice and simulated conditions associated with ovulatory wound repair, incessant ovulation, ovarian surface scarring, and aging. We found that the dominant site of cancer cell seeding was not the ovary but the nearby surgical wound site, which was associated with a strong and persistent inflammatory reaction. Conditions in the ovary associated with inflammation, such as acute ovulatory wound repair, active healing of the scarred ovarian surface, and mouse aging, contributed to increased seeding of the cancer cells to the surgical wound site and tissues surrounding the ovary. Changes in the ovary not accompanied by inflammation, such as completed ovulatory cycles and fully-healed scars on the ovarian surface, did not contribute to increased cancer cell seeding. We conclude that inflammation is the most likely mechanism by which ovulation and postmenopausal events contribute to the increased risk of ovarian cancer.

Despite modern day cytoreductive surgical techniques and combination chemotherapies for high-grade ovarian cancer, five-year survival rates remain below 40%[1]. However, when found early, the survival rate dramatically rises to 90%[1,2]. Thus, the ability to detect ovarian cancer in its earliest stages is critical to a cure. It is increasingly accepted that high-grade ovarian cancers actually originate in the fallopian tube with malignant cells shedding to the adjacent ovary[3–7]. Since the bulk of the tumor typically forms in the ovary, rather than the fallopian tube, ovaries must play a significant role in the early stages of cancer development. Discovering which cellular and molecular processes promote and inhibit the seeding of malignant cells to the ovary could facilitate the development of markers for early detection as well as the identification of rate-limiting events in the early stages of ovarian cancer development. If contextual molecular cues provided by the ovary are required for the clinical development of ovarian cancer, such molecules could serve as novel therapeutic targets to prevent cancer progression in the early stages, when cures are more viable.

Epithelial ovarian cancer is predominantly a disease of postmenopausal women[8]. Many theories of postmenopausal onset of ovarian cancer have been proposed, including incessant ovulation and inflammation, hormonal changes, reduced immunity, increased cell senescence, and uncontrolled production of reactive oxygen species[9–13]. Epidemiologic data consistently show that the risk of ovarian cancer increases with the number of ovulatory cycles[14–16], indicating that ovulation plays a significant role in ovarian cancer etiology. However, the peak incidence of menopause occurs at age 51, while the peak incidence of invasive epithelial ovarian cancer occurs at age 63[1]. Thus, most women develop ovarian cancer years after their last ovulatory cycle. Currently, it is unknown which conditions in the ovary promote tumor growth but the fact that more than 80% of ovarian cancer cases occur after menopause suggests that the events associated with menopause and aging are major contributing factors[8].

During the postmenopausal years, ovarian follicles are largely depleted and much of the remaining ovary is reduced to a collagenous scar tissue[17]. If the microenvironment of postmenopausal ovaries is conducive to the implantation of cancer cells, simulating postmenopausal conditions should result in more cancer cell deposits in the ovary. A better understanding of ovarian cancer pathogenesis, specifically the role of the early postmenopausal

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ovarian microenvironment in supporting the seeding and survival of malignant cells in the ovary, is necessary to develop strategies for ovarian cancer prevention and detection. Experiments in mice provide a convenient system in which both the effect and the outcome of specific conditions can be examined and quantified. Previously, we used a mouse model to study events associated with ovulation and ovulatory wound repair, including epithelial cell entrapment and the formation of epithelial inclusion cysts[18]. Here, we extended those studies by simulating various postmenopausal conditions in mice and quantifying cancer cell deposits for each condition. The goal of the study was to determine whether conditions associated with ovulation and aging increase the spread of cancer cells from the oviduct to the ovary. To account for a possible role of the immune system in ovarian cancer cell seeding, we used an immunocompetent FVB mouse model with syngeneic ovarian cancer cell aggregates implanted into the fallopian tube. Our data show that premenopausal and postmenopausal conditions contribute to increased cancer cell seeding only in the presence of an inflammatory reaction.

Materials and Methods

Cancer cell line. The FVB-syngeneic mouse ovarian cancer cell line, BR, was engineered with combinations of genetic alterations (p53-/-, Brca1-/-, myc, and Akt) as described[19]. We have shown that this ovarian cancer model recapitulates human serous histology, pattern of metastatic spread, and response to standard and targeted therapies[19–23]. The BR cells were subsequently transduced with luciferase lentiviral plasmid pLenti-CMVPuro-LUC (Addgene, w168-1) to generate BR-luc cells.

Preparation of cell aggregates. BR-luc cells were seeded at a density of 1×10^6 cells per well in Costar ultra-low attachment 6-well plates (Corning). The cells were incubated with 3 ml DMEM media in 5% CO_2 at 37 °C. After 2 days, culture media were collected in 15 ml conical tubes and cells were precipitated at 1000 rpm for 0.5 minutes. After two rounds of washing with phosphate buffered saline (PBS), large cell aggregates were separated into small aggregates by multiple pipetting through a 1 ml pipette tip.

Injection of cell aggregates into oviducts. All procedures in mice were performed in accordance with the approved Cedars-Sinai IACUC protocol (IACUC5318). The procedures were performed in an AAALAC-accredited facility at Cedars-Sinai Medical Center. The surgical procedures were performed according to the method described for embryo transfer into the oviduct (Manipulating the Mouse Embryo: A Laboratory Manual, 3rd Edition, ISBN-978-087969591-0). Under the dissecting microscope, a small incision between the infundibulum and the ampulla of the oviduct (equivalent to human fallopian tube) was created using Vannas scissors (Supplementary Video 1). The transfer pipette loaded with cell aggregates in PBS was inserted into the incision with the tip pointing toward the ovary and approximately 200 cell aggregates in 2 µl volume were injected into each oviduct (Supplementary Video 1).

Simulation of ovulatory and menopausal conditions. Mice were superovulated by intraperitoneal injection of pregnant mare serum (PMS) and human chorionic gonadotropin (hCG) as previously described[18]. In the control mice, PBS was injected instead of PMS and hCG. To generate scar tissue, bursa (a thin membrane covering the ovary in mice) was removed (Supplementary Video 2) and the ovarian surface was burned with a hand-held battery-powered cauterizer (Gemini Cautery System) (Supplementary Video 3).

Quantification of cancer cell deposits. Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation prior to harvesting the ovaries and surrounding tissues. To quantify macroscopic tumors, dimensions (length, width, height) were measured by calipers. Tumor volume (mm^3) was calculated using the equation $V = (L \times W \times H)/2$, where V is tumor volume, L is tumor length, W is tumor width, and H is tumor height. For the flat, superficial tumors that typically formed on the surgical wounds/scars, tumor area (mm^2) was measured using the equation $A = L \times W$, where A is tumor area size, L is tumor length, and W is tumor width. To quantify microscopic cancer cell deposits, the ovaries, oviducts, and surrounding fat tissues were fixed in formalin and embedded in paraffin. One 4 µm-thick section per sample was stained with hematoxylin and eosin (H&E) and evaluated under the light microscope for visible cancer cell deposits.

Statistical analyses. The statistical analyses were performed using GraphPad Prism (version 6.0; GraphPad Software). Intergroup differences were assessed by the Student's *t*-test.

Data availability. No datasets were generated or analyzed during the current study.

Results

Our ability to screen for early stage ovarian cancer is hampered by deficiencies in the understanding of the molecular and morphological steps involved in ovarian carcinogenesis. It is currently unknown why cancer cells in the fallopian tube have the propensity to migrate to the ovary where they tend to form a large tumor mass. To determine which ovarian conditions are most conducive to implantation of detached tubal cells, we simulated in mice conditions associated with ovulatory wound healing, incessant ovulation, atrophy/scarring, and aging.

Inflammatory events associated with ovulatory wound repair contribute to increased cancer cell seeding to tissues surrounding the ovary but are not directly associated with the implantation of cancer cells to the ovary. To simulate cancer cell seeding and entrapment during ovulatory wound healing, superovulation was induced in 4 week-old female FVB mice by intraperitoneal injection of PMS and hCG hormones (superovulated group, N = 6) or PBS (control group, N = 6). This combination of hormones induces ovulation of a large number of follicles to form 10–30 acute ovulatory wounds within one ovulatory cycle[18]. Two days after hCG (or control PBS) injection, when ovulatory wound repair is at its peak[18], cancer cell

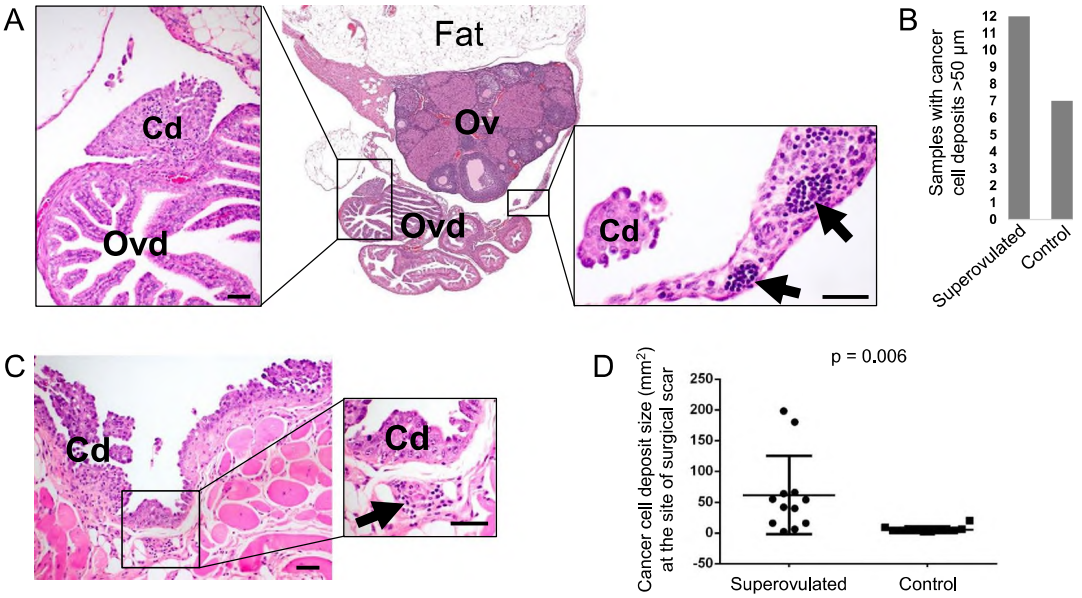
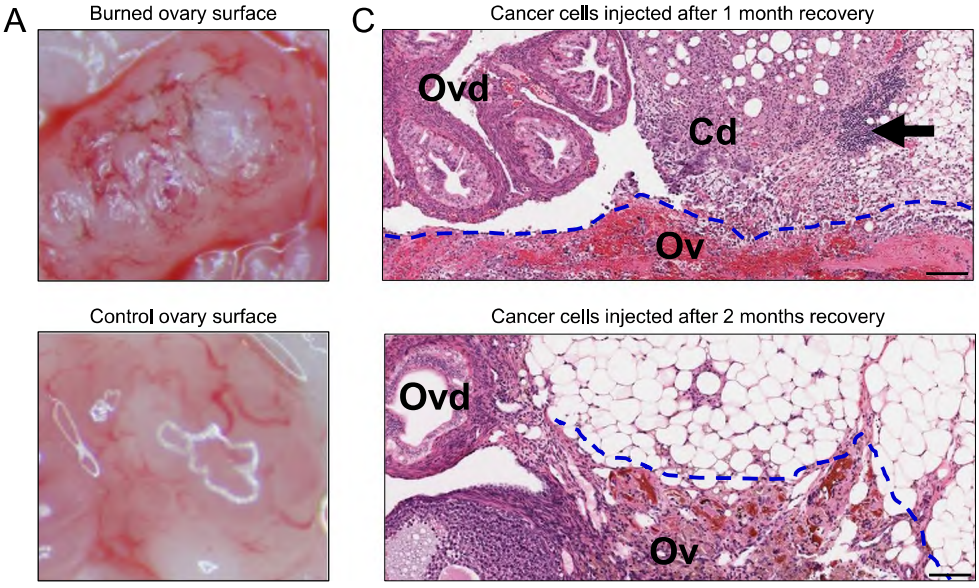


Figure 1. Assessing the effect of ovulatory wound repair on cancer cell seeding from the oviduct to the ovary and adjacent tissues. **(A)** Representative H&E-stained section of cancer cell deposits on the oviduct and ovary. Arrows indicate immune cell infiltrates. Size bars: 50 µm. Cd, cancer cell deposit; Ov, ovary; Ovd, oviduct. **(B)** Graph indicates the number of superovulated and control ovary/oviduct samples containing cancer cell deposits larger than 50 µm in diameter (out of 12 ovaries in each group). **(C)** H&E-stained section representing 'carpeting' of cancer cells along the surgical wound/scar site in the peritoneal wall. Arrow indicates an immune cell infiltrate. Size bars: 50 µm. Cd, cancer cell deposit. **(D)** Comparison of cancer cell deposit size at the surgical wound/scar site in superovulated and control mice.

aggregates were bilaterally implanted into the mouse oviduct. Three weeks later, intraperitoneal tumor dissemination was evaluated by recording the presence of ascites and measurable tumor deposits inside of the peritoneal cavity. Macroscopically visible swelling was observed in 4/12 ovaries from the superovulated mice and in 0/12 ovaries from the control mice. Microscopic cancer cell deposits in the oviducts, ovaries, and surrounding fat were quantified by pathologic examination of H&E-stained sections under the 4x objective, and the presence of cancer cells was further verified under higher magnification (Fig. 1A). These deposits in tissues surrounding the ovary were frequently associated with immune cell infiltrates (Fig. 1A). Cancer cell deposits larger than 50 µm were present in tissues surrounding the ovary (oviduct, bursa, and space between the fat and ovarian surface) in 12/12 samples from the superovulated mice and in 7/12 samples from the control mice (Fig. 1B). However, neither group of mice exhibited cell deposits directly on the ovarian surface or as intraovarian inclusions. These results suggest that ovulatory wound healing is not directly associated with the implantation of cancer cells to the ovary. In both groups of mice, the largest cancer cell deposits presented as carpeting of the abdominal wall at the sites of surgical wounds/scars (Fig. 1C). These surgical wound/scar cancer cell deposits were frequently associated with immune cell infiltrates (Fig. 1C) and were significantly larger in superovulated mice than in control mice (Fig. 1D). Taken together, our results indicate that events associated with ovulatory wound healing contributed to increased seeding of cancer cells to the surgical site and tissues surrounding the ovary. The lack of cancer cell deposits attached to the ovarian surface indicates that re-epithelialization of the ovarian surface does not significantly contribute to cancer cell seeding. It is more likely that ovulatory events contributed to increased inflammatory infiltrates, which attracted cancer cells and/or supported their survival and expansion.

Ovarian atrophy resulting from previous incessant ovulation is not associated with increased cancer cell seeding. To simulate cancer cell seeding in ovaries that endured repeated damage and repair due to multiple cycles of ovulation, six week-old female FVB mice were subjected to nine weeks of weekly intraperitoneal PMS and hCG hormone injections (repeatedly superovulated group, N = 7) or PBS injections (control group, N = 7). To mimic conditions in postmenopausal women whose ovaries have not been actively cycling for years, we waited 12 weeks after the last superovulation to implant BR-luc cell aggregates into the oviducts of the repeatedly superovulated and control mice. Eight weeks after cancer cell implantation, the tumor burden was evaluated in both groups. The majority of tumor deposits were found at the surgical wound/scar tissue, which was frequently fused with the adjacent fat and infiltrated with immune cells (data not shown). There were no macroscopically or microscopically visible cancer cell deposits on the ovaries and oviducts in either group of mice (data not shown). Thus, in the absence of acute inflammation, ovaries that have undergone repetitive superovulations do not appear to attract cancer cells any more than age-matched ovaries with a normal number of ovulatory cycles. One caveat to this experiment is that we did not achieve complete depletion of the oocytes pool despite nine cycles of superovulation, possibly because mice become unresponsive to hormone induction after reaching reproductive maturity[24].



B

	1 month recovery	2 month recovery
Mice with intraperitoneal tumors	8/8	7/7
Burned ovaries with > 50 µm cancer cell deposits	4/8	0/7
Control ovaries with > 50 µm cancer cell deposits	1/8	0/7

Figure 2. Assessing the effect of burn-induced ovary scarring on the seeding of intraperitoneally injected cancer cells. **(A)** Representative images of ovarian surface immediately after manipulation. Control ovaries were surgically released from the bursa while burned ovaries were first surgically released from the bursa, then superficially burned with a cauterizer. **(B)** Comparison of seeding efficiency of intraperitoneally injected cancer cells one or two months after surgical ovary manipulation. **(C)** Representative H&E-stained sections of cancer cell deposits on the ovaries and oviducts of mice that were injected one or two months after surgical ovary manipulation and euthanized four weeks later. Arrow indicates an immune cell infiltrate. Size bars: 100 µm. Cd, cancer cell deposit; Ov, ovary; Ovd, oviduct.

Burn-induced scarring of the ovarian surface is associated with increased cancer cell seeding to the ovaries and surrounding tissues only in the presence of active scar wound healing. To simulate events associated with postmenopausal ovary atrophy and connective tissue scarring, burn-induced scars were generated on the ovarian surface of the six week-old female FVB mice. In each mouse, one ovary was surgically released from the ovarian bursa (Supplementary Video 2) and superficially burned with a cauterizer (Fig. 2A and Supplementary Video 3). The contralateral ovary was surgically released from the bursa but not burned (control ovary) (Fig. 2A). Mice were intraperitoneally injected with a single-cell suspension of BR-luc cells (~1×10^6 cells) after one month recovery (N = 8) or two months recovery (N = 7). Four weeks after intraperitoneal cell injection, mice were euthanized for tumor burden quantification. Regardless of whether mice were intraperitoneally injected with cancer cells one month or two months after surgery, BR-luc cells formed multiple small tumor nodules on the mesothelial surfaces of the omentum, pancreas, diaphragm, spleen and abdominal lining; however, there were no visible tumor cell deposits on the surface of the burned or control ovaries. Therefore, we assessed microscopic cancer cell deposits in H&E-stained sections of ovaries/oviducts and adjacent fat. For the one month recovery group (N = 8), cancer cell deposits larger than 50 µm were present in the tissues surrounding the ovary (fat, oviduct, and bursa) in 4/8 burned ovaries and in 1/8 control ovaries (Fig. 2B). All ovaries that contained tumor deposits also had abundant immune cell infiltrates (Fig. 2C). For the two months recovery group (N = 7), none of the ovary sections contained cancer cell deposits (Fig. 2B). Although burn-induced scars on the ovarian surface were detectable two months later, the scars were no longer associated with abundant immune cell infiltrates (Fig. 2C). These results suggest that burn-induced scars attract cancer cells but only in the presence of inflammation.

Events associated with aging contribute to increased cancer cell seeding to the ovaries and surrounding tissues. BR-luc cancer cell aggregates were bilaterally implanted into the oviducts of eight week-old (N = 10) and greater than one year-old (age range 14–19 months; N = 10) female FVB mice. Mice were euthanized for analysis four weeks after cancer cell implantation. Both groups of mice developed multiple intraperitoneal metastases with the largest tumor masses present on the omentum and abdominal wall. Omental and abdominal wall masses were more frequent in aged mice (Fig. 3A). Three of the aged mice also exhibited unilateral or bilateral uterine horn hyperplasia (data not shown). H&E-stained sections showed that the ovaries from young mice contained multiple follicles in different phases of maturation (data not shown), while the ovaries from old mice were devoid of follicles (Fig. 3B). Microscopic examination of the ovaries and adjacent tissues (oviduct,

A

	8 week-old	>1 year-old
Mice with omental tumor masses >0.5 cm	1/10	3/10
Mice with abdominal wall tumor masses >0.5 cm	3/10	5/10
Ovaries with > 50 µm cancer cell deposits	6/17	11/15

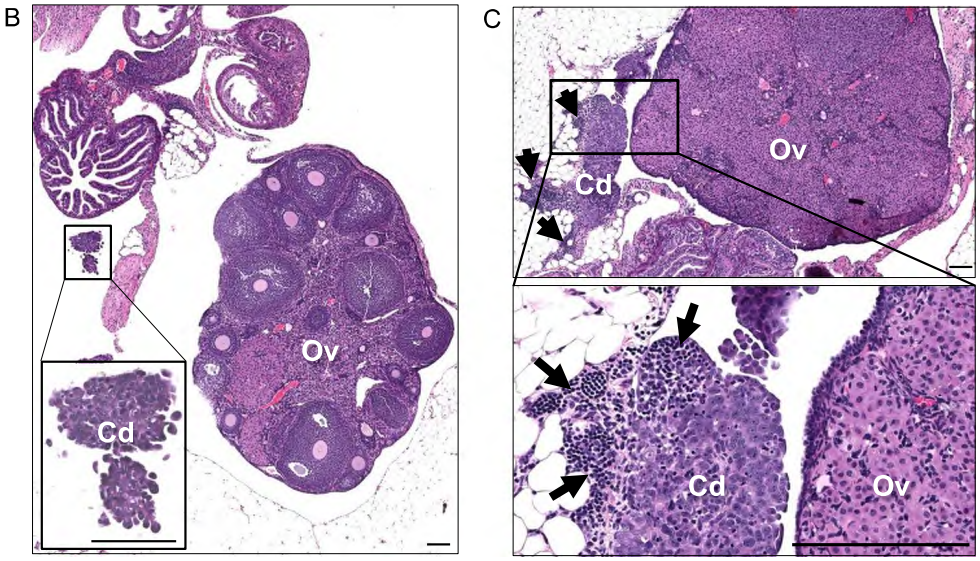


Figure 3. Assessing the effect of aging on cancer cell seeding from the oviduct to the ovary and adjacent tissues. **(A)** Comparison of seeding efficiency of cancer cells implanted into the oviducts of young (8 weeks) and aged (>1 year) mice. In H&E-stained sections, several ovaries were excluded from the analysis because they were either missing from the slide section or the tissue was insufficient for evaluation. **(B,C)** Representative H&E-stained section of cancer cell deposits on ovaries and oviducts four weeks after surgical implantation of cancer cells into the oviducts of (**B**) 8-week-old mice and **(C)** >1-year-old mice. Arrow indicates an immune cell infiltrate. Size bars: 100 µm. Cd, cancer cell deposit; Ov, ovary.

bursa, and adjacent fat) revealed that tumor cell deposits were more frequent in aged mice (Fig. 3A), which also contained more abundant immune cell infiltrates (Fig. 3B). These results suggest that ovaries from aged mice are more conducive to cancer cell seeding than ovaries from young mice.

Discussion

A poor understanding of the initiating events in ovarian cancer has significantly hampered our efforts towards early ovarian cancer detection and prevention. Most early stage cancers in the tubal fimbria are associated with a dominant mass in the ovary, indicating that the ovarian microenvironment is essential for tumor growth. However, conditions that promote cancer cell seeding and growth in the ovary are still unknown. Recently, Yang-Hartwich and colleagues used a mouse xenograft model to test the role of ovulatory wound repair in the migration of cancer cells from the injection site in the uterus toward the ovary[25]. Consistent with epidemiologic data that increased ovulation is strongly associated with ovarian cancer[15,16], they showed that superovulation in mice enhances the migration and adhesion of malignant cells to the ovary and that this attraction is mediated through the release of cytokines/chemokines from the surface wound created by oocyte release[25]. Using a syngeneic immunocompetent mouse model with cancer cells surgically implanted into the oviduct, we confirmed that superovulation contributes to ovarian cancer cell seeding. Tumor cell deposits were accompanied by immune infiltrates, indicating that ovulation-induced inflammation may play an important role in cancer cell seeding. It is possible that the inflammatory reaction is the only factor that contributes to increased cancer cell seeding because the largest cancer cell deposits typically formed in the abdominal wall along surgical wounds, which were associated with extensive immune infiltrates. It appears that the wounded surface of the superovulated ovary did not play a direct role in cancer cell attraction as there were no cancer cells attached to the ovarian surface epithelium or inside the ovarian stroma. The importance of the inflammatory reaction, rather than the damaged ovarian surface in cancer cell seeding, was illustrated by the next two sets of experiments in which we repeatedly wounded the ovarian surface by multiple rounds of superovulation or burned the ovarian surface to induce scarring. The wounded/scarred ovarian surface proved to be attractive to cancer cells only if the wounds were 'fresh'. If ovarian wounds/scars were allowed to recover for two months, cancer cells were no longer attracted to the ovarian surface but were still attracted to other sites in the peritoneal cavity where inflammation persisted. It is well established that aging is characterized by subclinical, chronic inflammation[26]. Consistent with multiple studies showing that the overall proinflammatory status in older mice is associated with increased tumor burden[27], our results show that oviductal implantation of cancer cells in aged mice resulted in increased tumor burden throughout the peritoneal cavity.

Our finding that surgical wounds in mice attract cancer cells is consistent with an observation in clinical practice that wound trauma in patients is associated with cancer recurrence[28,29]. It has been shown that an early peak of breast cancer recurrence is due to surgery-driven intervention[30]. The exact reasons for surgery-related cancer

attraction are not fully understood but possible factors include surgery-related acute wound healing process, inflammation, and activation of dormant cancer cells by surgery-driven growth factors[31–33]. If inflammation is a key factor in cancer cell seeding, what are the contributions of other factors strongly associated with increased cancer incidence, such as ovulation, oocyte depletion and atrophy, and aging? Our data in a mouse model are consistent with the concept that most of the factors implicated in ovarian cancer incidence converge on inflammation as a common denominator. One successful path to ovarian cancer prevention has been controlling factors that induce inflammation, such as the use of oral contraceptives to suppress ovulation[34]. Epidemiologic data show that aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) can be beneficial in the prevention of multiple cancers, including ovarian[35,36]. Although factors associated with the increased risk of ovarian cancer, such as aging and menopause cannot be prevented, the risk can be reduced by suppressing inflammation. The results of our study in a mouse model confirm previous results that inflammation is a key factor in promoting ovarian cancer cell seeding. An understanding of the mechanisms by which inflammation plays a role in the early stages ovarian cancer will be necessary for effective ovarian cancer prevention.

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Author Contributions

D.J. performed all experiments described in this study except mouse surgery, which was performed by Y.N. and MK. D.J. also participated in data collection, analysis, and interpretation. S.O. conceived and designed the study, participated in data analysis and interpretation, and wrote the manuscript. All authors contributed to manuscript revisions and approved the submitted version.

Additional Information

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